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Summary of Information

Version Number	Version Date	Current Working Copy
1	Fri Sep 29 07:09:45 EDT 2006	N
2	Fri Sep 29 07:09:45 EDT 2006	N
3	Fri Sep 29 07:09:45 EDT 2006	N

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us-10-773-387-4.n2p.rup	Download

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	6	estell.in. and bacillus and ((cysteine or cystine) adj (protease or proteinase))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:31
L2	2358	bacillus and ((cysteine or cystine) adj (protease or proteinase))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:27
L3	2336	l2 and (inactivat$ or attenuat$ or (knock adj out) or knock-out or delet$ or mutat$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:30
L4	2323	l2 and (inactivat$ or attenuat$ or (knock adj out) or knock-out or delet$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:28
L5	2057	l4 and subtilis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:28
L6	5	l5 and cp3	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:46
L7	4	(cysteine adj endopeptidase) same (inactivat$ or attenuat$ or (knock adj out) or knock-out or delet$ or mutat$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:30
L8	491	((cysteine or cystine) adj (protease or proteinase)) same (inactivat$ or attenuat$ or (knock adj out) or knock-out or delet$ or mutat$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:31
L9	133	l8 and (bacillus adj subtilis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:31
L10	290	(mannose-6-phosphate adj isomerase) OR (phosphomannose adj isomerase)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:43
L11	38	l10 same subtilis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:43

EAST Search History

L12	0	l11 and muri	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:43
L13	4	l11 and pmi	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:43
L14	6	pmi same subtilis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:44
L15	11	l5 and cp2	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2006/09/29 07:46

Search Results for 10/773,387

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     (FILE 'HOME' ENTERED AT 07:35:22 ON 29 SEP 2006)

     FILE 'REGISTRY' ENTERED AT 07:37:27 ON 29 SEP 2006
                E "CYSTEINE PROTEASE"/CN 25
L1            1 S E3
                E "CYSTEINE ENDOPEPTIDASE"/CN 25
L2            1 S E3
                E "PHOSPHOMANNOSE ISOMERASE"/CN 25
L3            1 S E3

     FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, WPIDS' ENTERED AT
     07:38:53 ON 29 SEP 2006
L4        14289 S L1 OR L2 OR L3
L5           24 S L4 AND (BACILLUS (W) SUBTILIS)
L6           19 DUP REM L5 (5 DUPLICATES REMOVED)

     FILE 'CAPLUS' ENTERED AT 07:40:39 ON 29 SEP 2006
                E ESTELL D/AU 25
L7            4 S (E4 OR E5 OR E6 OR E7 OR E8 OR E9) AND (L1 OR L2 OR L3)

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/14529

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N1/20 C12N1/21 C11D3/386
//C12N9/56,C12N9/90,(C12N1/20,C12R1:07)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 89 10976 A (PUBLIC HEALTH RESEARCH INST OF) 16 November 1989 see the whole document ---	1,13
X	MARGOT P ET AL: "The gene of the N-acetylglucosaminidase, a Bacillus subtilis 168 cell wall hydrolase not involved in vegetative cell autolysis." MOLECULAR MICROBIOLOGY, (1994 MAY) 12 (4) 535-45. JOURNAL CODE: MOM. ISSN: 0950-382X., XP002084429 ENGLAND: United Kingdom see figure 3 -& EMBL/GENBANK DATABASES Accession no P39841, Sequence reference MANU_BASCU, 01-February 1995 "Mannose-6-phosphate isomerase" XP002084431 --- -/--	11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1998	01.03.99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	VAN DER SCHAAL C.A.

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SADAIE Y ET AL: "Nucleotide sequence and analysis of the phoB-rrnE-groESL region of the Bacillus subtilis chromosome" MICROBIOLOGY, vol. 143, June 1997, pages 1861-1866, XP002084430 see table 1 -& EMBL/GENBANK DATABASES Accession no D88802, Sequence reference BSD802, 22 April 1995 "ydhs" XP002084432	11
A	EP 0 369 817 A (BIOTEKNIKA INTERNATIONAL) 23 May 1990 see the whole document	15-17
P,X	KUNST F ET AL: "The complete genome sequence of the Gram-positive bacterium Bacillus subtilis" NATURE, vol. 390, 20 November 1997, pages 249-256, XP002080813 see tables 1,II.1.1	11

Microbiology (1994), 140, 471–478 Printed in Great Britain

The *gerB* region of the *Bacillus subtilis* 168 chromosome encodes a homologue of the *gerA* spore germination operon

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Spores of *gerB* spore germination mutants of *Bacillus subtilis* 168 are defective in response to the germinative mixture of L-asparagine, glucose, fructose and potassium ions (AGFK), but are normal in the L-alanine (ALA) triggered germination response. A λ clone of 15 kbp carrying the *gerB* region has been identified. Sequencing of the *gerB* region of the clone revealed a cluster of three ORFs encoding putative proteins of 53·3, 41·3 and 42·4 kDa (GerBA, GerBB and GerBC, respectively). The first two of these proteins have substantial hydrophobic regions and the third is a possible lipoprotein. At least two, and probably all three products are required for normal germination in AGFK. The three proteins form a set of homologues of the products of the *gerA* operon, mutations in which cause a defect in the ALA germination pathway, but cause no defect in AGFK. The GerB proteins show 42%, 31% and 35% identity at the amino-acid level to the corresponding GerA proteins, and the homologues occur in the same order in both operons.

Keywords: *Bacillus subtilis*, sporulation, germination, *gerB*, membrane protein

INTRODUCTION

Spores of *Bacillus subtilis* 168 have two known germination reponses, which have been well-characterized: they germinate in response to alanine alone (ALA) or to the mixture of asparagine, glucose, fructose and potassium ions (AGFK). Spore germination (*ger*) mutants have been isolated which are defective in either or both of these responses (Moir & Smith, 1990), which suggests that there are separate germinant-specific triggers, with a later convergence of the germination pathways.

Of all the *ger* mutants the only ones with a specific defect in ALA germination result from mutation in the *gerA* operon (Moir & Smith, 1990). This is a tricistronic operon which encodes proteins of 53·3, 41·3 and 42·4 kDa, all of which could be membrane-associated (Feavers *et al.*, 1985; Zuberi *et al.*, 1987). The *gerA* operon is expressed in the forespore compartment of the developing sporangium from a promoter recognized by $E\sigma^G$, and it has been suggested that the proteins form a complex at the inner spore membrane which acts as a receptor for alanine (Feavers *et al.*, 1990).

Mutants of *gerB* and *gerK* have defects specifically in the AGFK response but retain a normal ALA response (Moir *et al.*, 1979; Irie *et al.*, 1982) and *gerK* has been associated with the glucose component of the response on account of the inability of glucose to improve the ALA response in *gerK* mutants under certain conditions (Irie *et al.*, 1982). This effect is not seen in *gerB* mutant spores (McCann, 1989). Mutants of *gerD* appear to be defective in both germination pathways to some extent (Moir *et al.*, 1979); however the product of *gerD* may be associated with the fructose component of the response (Irie *et al.*, 1986). The *gerB* mutations have been located by phage PBS1-mediated three-factor transductional crosses to 314 degrees on the *B. subtilis* 168 chromosome. Mutations are not known to affect any one specific component of the AGFK response.

Molecular genetical analysis of several *ger* genes has as yet shed little light on the possible mechanism of germination (Moir & Smith, 1990). The predicted proteins generally

Abbreviations: ALA, L-alanine; AGFK, L-asparagine; glucose, fructose and K^+ ions (germinative mixture).

The GenBank accession number for the nucleotide sequence reported in this paper is L16960.

lack characteristic motifs and database searches reveal no homology with other proteins.

Although *tag* (teichoic acid biosynthesis) and *gerB* have not been mapped relative to one another, available data place them near to each other on the chromosome, with *gerB* on the *ori*-proximal side (Piggot *et al.*, 1990). We have identified a λEMBL4 derivative carrying *gerB* from a series of overlapping clones of the *tag–gta* region. Sequence information indicating the possible nature of the gene products, reported here, has provided more essential information in the attempt to identify the mechanism of spore germination.

METHODS

Bacterial strains and media. Strains of *B. subtilis* 168 and *Escherichia coli* K12 used are listed in Table 1. *B. subtilis* was cultured on nutrient agar (NA), and *E. coli* on Luria–Bertani agar (LA), with appropriate selective conditions as described by Yon *et al.* (1989). Transformation of *B. subtilis* was by the method of Anagnostopoulos & Spizizen (1961) and of *E. coli* by the method of Mandel & Higa (1970). Ger phenotypes were scored on plate tests as described by Irie *et al.* (1982).

DNA manipulation. Plasmid and chromosomal DNA preparation, restriction, ligation and gel electrophoresis were carried out as described by Sammons *et al.* (1987). All subcloning was done using pMTL20EC in DH5α. λ DNA preparation was as described by Maniatis *et al.* (1982), using *E. coli* P2392 as a host. Purification of restriction fragments from gels was achieved using Geneclean (Bio101).

DNA sequencing. Restriction fragments of 150–1500 bp were prepared from parent subclones, gel-purified, subcloned into pMTL20EC and sequenced. M13 universal, reverse and −40 primers were used wherever possible, supplemented by a small number of custom primers. Double-stranded dideoxy sequencing was done using the Sequenase system as recommended by the manufacturers (USB). Electrophoresis was carried out using 4–8% (w/v) denaturing acrylamide gels in a BRL S0 apparatus (Gibco).

Table 1. Bacterial strains

Strain	Genotype	Source
B. subtilis		
L5047	*pheA1 purA16 hisA35 trpC2 metB5*	D. Karamata
1604	*trpC2*	Lab. stock
4688	*gerB18 trpC2*	Lab. stock
4950	*gerB90 trpC2*	Lab. stock
4952	*gerB92 trpC2*	Lab. stock
4954	*gerB94 trpC2*	Lab. stock
5182	*gerB15 trpC2*	Lab. stock
5301	*gerB18 pheA1 purA16 hisA35 trpC2*	This study
5304	*gerB15 pheA1 purA16 hisA35 trpC2*	This study
E. coli		
DH5α	F⁻ *endA1 hsdR17*(r^- m^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF–lacZYA*) *U169* ϕ80d *lacZΔM15*	D. Karamata (Lausanne)
P2392	F⁻ *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* lysogenic for P2	D. Karamata

RESULTS

Identification of a *gerB* clone and sequencing

Mauël *et al.* (1989) identified clones carrying the *tag* region in a λEMBL3 bank and Young *et al.* (1989) subsequently extended the cloned region by chromosome-walking using λEMBL3 and λEMBL4 banks. As *gerB* was thought to be on the *ori*-proximal side of *tag*, the appropriate clone, λE51, was screened for the presence of *gerB*. As Ger^+ phenotypes cannot be selected directly, *gerB pheA* derivatives of L5047 were made for screening purposes. DNA from strains carrying either the *gerB15* or the *gerB18* allele, 5182 and 4688, respectively, was used to transform strain L5047. Selection was for Met^+ and transformants were screened to find strains with a Ger^- phenotype. Appropriate double mutants, strains 5304 and 5301, were transformed with a mixture of 0·5 μg DNA from a λ clone carrying Phe^+ DNA and 5 μg λE51. One hundred and fifty transformants from each cross were screened for their Ger phenotype using a modification of the tetrazolium test (Irie *et al.*, 1982) and it was found that in congression experiments with the λE51 DNA present both the *gerB* alleles were corrected, with about 50% of the Phe^+ transformants having a Ger^+ phenotype. Congression experiments with λE51-derived plasmid subclones in place of λE51 revealed the approximate location of two of the *gerB* alleles within the clone (Fig. 1).

The sequence of the *gerB* region was generated by dideoxy sequencing of small restriction fragments subcloned in plasmid vector pMTL20EC (Chambers *et al.*, 1988) as described in Methods. All restriction sites were overlapped in the sequence.

Computer analysis of the sequence

The sequence of 3·8 kb was analysed using the UWGCG package, version 7.1 (Devereux *et al.*, 1984) except where otherwise stated. Three large adjacent ORFs were identified by direct translation and were confirmed by codon usage analysis (data not shown). The sequence and putative translations are shown in Fig. 2. ORFs 1, 2 and 3 have been named *gerBA*, *gerBB* and *gerBC*, respectively. There are three possible initiation (start) codons for *gerBB*, a GTG and ATG which overlap the *gerBA* termination (stop) codon, and an ATG which is 5 bp downstream. The last has a possible Shine–Dalgarno sequence, although it is weak and close to the ATG. There is an overlap between the probable stop and start codons of *gerBB* and *gerBC*. All the ORFs have potential ribosome-binding sites with homology to the 3′ end of the *B. subtilis* 16S RNA molecule (Stewart & Bott, 1983) appropriately

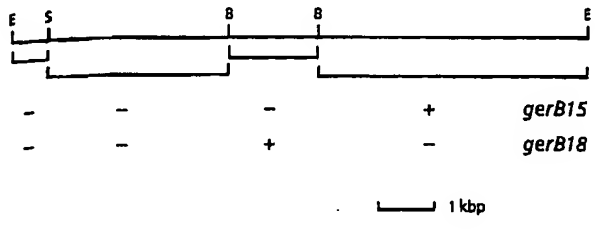

Fig. 1. Marker correction with λE51. Restriction map of the insert in λE51 and four plasmid subclones derived from it. The five clones were used in congression experiments (see text) to localize the position of the *gerB15* and *gerB18* alleles in the insert. B, *Bam*HI; E, *Eco*RI; S, *Sph*I; +, correction to Ger$^+$; −, no correction of Ger$^-$ phenotype.

located in front of their start codons (see Fig. 2). This arrangement suggests that the *gerB* mutations may identify an operon. There is no obvious terminator after *gerBC*, but no start of another ORF.

The predicted molecular masses of GerBA, GerBB and GerBC are 53·971, 41·709 and 42·468 kDa, respectively. Database searches using the UWGCG Fasta program revealed extensive homology between the polypeptides encoded by *gerB* and those encoded by the *gerA* operon as shown in UWGCG Gap comparisons (Fig. 3a–c). The homology exists across the full length of the genes' products and all the pairs of proteins are of similar size. GerBA and GerAA are 42% identical at the amino acid level, and show 66% similarity, taking conservative substitutions into account, of the primary sequence. Hydroplots (Kyte & Doolittle, 1982) show a central hydrophobic domain sandwiched between a large hydrophilic domain at the N-terminus and a smaller one at the C-terminus of GerBA (Fig. 4a). The polypeptides are well-conserved at the functional level, but the hydrophobic domain shows the greatest level of identity. This is unusual, as hydrophobic regions are generally conserved for functionality rather than identity (e.g. Hiles *et al.*, 1987; Higgins *et al.*, 1990).

A further possible member of the GerAA/GerBA group is the *spoVAF* gene product. This ORF was partially sequenced by Fort & Errington (1985) as the sixth predicted member of the *spoVA* operon and the predicted N-terminal sequence had homology to GerAA. When the sequence of the downstream gene, *lysA*, was published, the upstream sequence included the second half of *spoVAF* (Yamamoto *et al.*, 1991; Sorokin *et al.*, 1993) so that the complete sequence is now available. UWGCG Gap comparisons of GerAA and GerBA with the predicted SpoVAF polypeptide showed a lower degree of conservation than exists between GerAA and GerBA (data not shown), although the polypeptide is still significantly homologous, with around 25% identical residues and over 50% similarity taking conservative substitutions into account. SpoVAF is also of a similar size (462 amino acid residues) to GerAA and GerBA. However, insertional inactivation of *spoVAF* has no known effect upon spore germination (E. H. Kemp, personal communication). There are no further ORFs downstream of *spoVAF* in the putative operon.

The hydroplot of GerBB (Fig. 4b) shows that it is predominantly hydrophobic with no large hydrophilic regions. There is homology across the whole length of the GerAB/GerBB polypeptides but, as is common for hydrophobic proteins, this is at the functional (60% conservative substitutions) rather than identical (30% identity) level.

The hydrophobic domains of both GerBA and GerBB are similar in size to their GerA homologues, but their hydroplots are not as clearly defined as for GerAA and GerAB, making estimation of the number of membrane-spanning helices difficult on the basis of these data. However the close similarity with the *gerA* products, and the clarity of the hydroplots of the latter, could suggest that GerBA and GerBB have seven and 10 or 11 transmembrane regions, respectively. If this is correct then the hydrophilic domains of GerBA would be on opposite sides of the membrane.

GerBC is predominantly hydrophilic, but has a small hydrophobic region at the N-terminus which has homology to the signal sequence for exported prokaryotic lipoproteins (Yamaguchi *et al.*, 1988). GerBC shows 35% identity with its homologue, and 58% conservative substitution.

Upstream of the *gerBA* translational start is a potential binding site for σ^G, the sigma factor which directs gene expression in the forespore after engulfment by the mother cell (Fajardo-Cavazos *et al.*, 1991). This may indicate a further degree of homology between the two gene clusters, as the *gerA* operon is a known member of the σ^G regulon (Feavers *et al.*, 1990). Between bases 10 and 20 of this sequence is the −35 region for ORFX, a putative gene in the opposite orientation to *gerB* in the upstream sequence (Margot, 1992). ORFX lies adjacent to *lytD* (glucosaminidase) which in turn is adjacent to the *tagABC* operon (Margot, 1992).

Localization of the *gerB* alleles

Strains carrying *gerB* alleles were transformed with integrative plasmids carrying regions of the putative *gerB* operon. Three subclones were used: A carried most of *gerBA*, and the upstream region; B carried an overlap between *gerBA* and *gerBB*; and C carried an overlap between *gerBB* and *gerBC*. The results of the integrations are shown in Fig. 5. The integration of subclone C into wild-type gives a TZM^W (Ger$^-$) phenotype. This indicates that the *gerBC* product must be required for germination. This result for subclone C is an argument for operon structure. There is a complete copy of the *gerBC* ORF on the chromosome after the integration event, but a Ger$^-$ phenotype is obtained. Therefore the ORF must be separated from its promoter by at least the 250 bp which are present between the start of the subclone and the start

```
      HindIII                    -35                    -10
    1 AAGCTTTGTTTCAGCCTTTTCCTCGATAAGAATAATTCTCCTTTTTTGATACAAATTAATAAAAACCGTCAATATGTTTAAGAAGGAGAGAAACTATGCA
                                                                                                  M  Q

  101 AATCGACTCTGATCTCCAGAACAATTTAGACACACTGAAAAAAACATTGGGACAAAACGACGATATGATGTTTTATACATTTGCTTTCGGAGATAGCAGA
        I  D  S  D  L  Q  N  N  L  D  T  L  K  K  T  L  G  Q  N  D  D  M  M  F  Y  T  F  A  F  G  D  S  R

  201 CAAAAGGCGTGTTTACTGTATATTGACGGTCTGACAGAGAATAAAATGCTGGCGCAATACGTCATTTCTCCTTTACAAAAAGAGGCGTTGGCCCATAAGG
        Q  K  A  C  L  L  Y  I  D  G  L  T  E  N  K  M  L  A  Q  Y  V  I  S  P  L  Q  K  E  A  L  A  H  K  E

  301 AATGCTCGATTGAAGACTTATCCGCCTTTTTTTTCGGCTTTCACCACAGCGTTGTTTCTACAATGAAAGAAATAGAGCAGCTCGTTTTTTCAGGGCAAGC
          C  S  I  E  D  L  S  A  F  F  F  G  F  H  H  S  V  V  S  T  M  K  E  I  E  Q  L  V  F  S  G  Q  A

  401 CATTTTGCTCGCTGATGGCTATCGCGGAGGATTAGCTTTCGACACAAAGTCAGTCGCAACACGCAGTCTTGATGAACCCTCCTCCGAAGTCGTGGAACGC
        I  L  L  A  D  G  Y  R  G  G  L  A  F  D  T  K  S  V  A  T  R  S  L  D  E  P  S  S  E  V  V  E  R

  501 GGCCCTAAAATAGGATTTATTGAAAAGCTGCGGACAAACACGGCGCTTTTGCGTGAACGGACGAGTGATCCTAATCTCGTCATCAAAGAAATGACACTCG
        G  P  K  I  G  F  I  E  K  L  R  T  N  T  A  L  L  R  E  R  T  S  D  P  N  L  V  I  K  E  M  T  L  G

  601 GAAAAAGGACCAAAAAGAAAATCGCTGTCGCTTATATTCAGGATATCGCCCCAGATTATGTCGTCAAAGAAGTGTTTAAAAGGCTGAAATCAGTCAATAT
        K  R  T  K  K  K  I  A  V  A  Y  I  Q  D  I  A  P  D  Y  V  V  K  E  V  F  K  R  L  K  S  V  N  I

  701 AGATAATTTGCCGGAATCGGGGACGCTTGAACAGCTGATTGAAGACGAACCATTTTCAATTTTCCCCACAATATTAAGCACAGAACGACCGGACCGGGTC
        D  N  L  P  E  S  G  T  L  E  Q  L  I  E  D  E  P  F  S  I  F  P  T  I  L  S  T  E  R  P  D  R  V

  801 GAAAGTTCTCTATTAGAAGGGCGAGTGTCCATATTAGTGGATGGTACGCCGTTTGCTTTGATTGTACCGGCTACGGTTGACGAATTTATTCATTCGCCCG
        E  S  S  L  L  E  G  R  V  S  I  L  V  D  G  T  P  F  A  L  I  V  P  A  T  V  D  E  F  I  H  S  P  D
                    BamHI
  901 ATGATTACAGCCAGCGCTGGATCCCAATGTCACTTGTACGCCTTCTTCGTTATTCCAGCATCCTGATCACCATTTATTTGCCCGGTCTGTATATTTCTCT
          D  Y  S  Q  R  W  I  P  M  S  L  V  R  L  L  R  Y  S  S  I  L  I  T  I  Y  L  P  G  L  Y  I  S  L

 1001 CGTTTCTTTTCACACCGGGCTATTGCCGACTAGAATGGCCATTTCCATTGCAGGCAGCAGGCTTAACGTACCATTCCCGCCTTTTGTAGAGGCCTTTATC
        V  S  F  H  T  G  L  L  P  T  R  M  A  I  S  I  A  G  S  R  L  N  V  P  F  P  P  F  V  E  A  F  I

 1101 ATGATCTTTACGATCGAATTGATTCGAGAAGCCGGATTAAGGCTGCCTAAGCCGATTGGACAGACAATTGGCCTTATCGGCGGCGTTGTCATCGGACAGG
        M  I  F  T  I  E  L  I  R  E  A  G  L  R  L  P  K  P  I  G  Q  T  I  G  L  I  G  G  V  V  I  G  Q  A

 1201 CAGCTGTTCAGGCGCAAATTGTCAGTGCGCTTATGGTCATTGTCGTTTCTGTCACAGCACTGGCATCCTTTACCGTTCCTTCATACGCCTATAACTTTCC
        A  V  Q  A  Q  I  V  S  A  L  M  V  I  V  V  S  V  T  A  L  A  S  F  T  V  P  S  Y  A  Y  N  F  P

 1301 GCTGCGGATCATTCGGATCGGGGTTATGATAAGTGCAACAGCGCTTGGCATGTACGGCGTTATAATGGTTTATCTGTTTGTGATCGGCCATCTCATGCGC
        L  R  I  I  R  I  G  V  M  I  S  A  T  A  L  G  M  Y  G  V  I  M  V  Y  L  F  V  I  G  H  L  M  R
          HindIII
 1401 CTGAAAAGCTTGGCCAGGATTACATTATCCGATCATGCGCAGCCTGGACAGGATTTGAAAGACACAGTCATCCGTATTCCCACGATGTTTTTAAAAAGAA
        L  K  S  L  A  R  I  T  L  S  D  H  A  Q  P  G  Q  D  L  K  D  T  V  I  R  I  P  T  M  F  L  K  R  R

 1501 GACCGACACGAAACGATCCCGAAGATAACATCAGACAAAGGTGATGGCTATGAGGAAATCAGAGCATAAACTGACATTTATGCAGACGCTCATTATGATC
          P  T  R  N  D  P  E  D  N  I  R  Q  R  *        M  R  K  S  E  H  K  L  T  F  M  Q  T  L  I  M  I

 1601 AGCAGCACATTGATTGGTGCCGGGGTGCTGACCCTTCCCCGCTCAGCCGCCGAAACCGGCAGTCCGAGCGGATGGCTAATGATACTGCTCCAGGGCGTTA
        S  S  T  L  I  G  A  G  V  L  T  L  P  R  S  A  A  E  T  G  S  P  S  G  W  L  M  I  L  L  Q  G  V  I

 1701 TTTTTATTATCATCGTTCTGCTTTTTTTGCCTTTTCTTCAAAAAAACAGCGGAAAAACTCTTTTTAAGCTCAACAGCATTGTAGCTGGGAAATTCATCGG
        F  I  I  I  V  L  L  F  L  P  F  L  Q  K  N  S  G  K  T  L  F  K  L  N  S  I  V  A  G  K  F  I  G

 1801 CTTTCTATTGAATTTATATATCTGTCTATATTTCATTGGGATTGTTTGCTTTCAAGCTCGGATTTTGGGAGAGGTTGTCGGATTCTTTTTGTTGAAAAAT
        F  L  L  N  L  Y  I  C  L  Y  F  I  G  I  V  C  F  Q  A  R  I  L  G  E  V  V  G  F  F  L  L  K  N

 1901 ACCCGAATGGCAGTTGTGGTGTTTATATTTCTTGCAGTTGCCATCTATCATGTAGGCGGAGGCGTTTATTCAATTGCAAAAGTATACGCTTATATTTTTC
        T  R  M  A  V  V  V  F  I  F  L  A  V  A  I  Y  H  V  G  G  G  V  Y  S  I  A  K  V  Y  A  Y  I  F  P
                                                                                                  HindIII
 2001 CTATAACCCTTATTATTTTTATGATGCTTCTGATGTTCAGCTTTCGCTTGTTCCAGCTTGATTTTATCCGGCCGGTATTTGAAGGAGGCTATCAAAGCTT
        I  T  L  I  I  F  M  M  L  L  M  F  S  F  R  L  F  Q  L  D  F  I  R  P  V  F  E  G  G  Y  Q  S  F

 2101 TTTCTCTTTATTCCCAAAAACATTATTATATTTCTCCGGATTTGAAATCATTTTTTACCTGGTCCCCTTTATGAGAGATCCAAAGCAAGTGAAAAAGGCT
        F  S  L  F  P  K  T  L  L  Y  F  S  G  F  E  I  I  F  Y  L  V  P  F  M  R  D  P  K  Q  V  K  K  A

 2201 GTTGCTCTGGGCATCGCGACTTCCACATTGTTCTACAGCATTACTTTGCTCATTGTGATTGGCTGTATGACTGTGGCTGAGGCAAAAACGGTGACATGGC
        V  A  L  G  I  A  T  S  T  L  F  Y  S  I  T  L  L  I  V  I  G  C  M  T  V  A  E  A  K  T  V  T  W  P

 2301 CGACCATTTCCCTTATTCACGCATTAGAGGTTCCGGGTATTTTTATTGAACGATTTGATTTGTTCTTACAGCTGACCTGGACAGCCCAGCAATTTGCCTG
        T  I  S  L  I  H  A  L  E  V  P  G  I  F  I  E  R  F  D  L  F  L  Q  L  T  W  T  A  Q  Q  F  A  C
          BamHI
 2401 TATGCTCGGATCCTTTAAAGGCGCCCATATCGGGTTAACGGAAATCTTTCATCTAAAAAATAAAAACAATGCATGGCTGCTGACTGCAATGCTGGCTGCG
        M  L  G  S  F  K  G  A  H  I  G  L  T  E  I  F  H  L  K  N  K  N  N  A  W  L  L  T  A  M  L  A  A

 2501 ACGTTTTTCATAACGATGTATCCCAAGGACTTGAATGACGTGTTTTATTACGGGACGCTTCTTGGATATGCTTTCTTAATTGTGATTACAATTCCATTCT
        T  F  F  I  T  M  Y  P  K  D  L  N  D  V  F  Y  Y  G  T  L  L  G  Y  A  F  L  I  V  I  T  I  P  F  F

 2601 TCGTCTGGTTTTTAAGCTGGATACAGAAAAAAATAGGGAGGGGGCAGCTGCAATGAAAACAGCATCAAAATTCTCCGTCATGTTTTTTATGCTGCTGGCC
        V  W  F  L  S  W  I  Q  K  K  I  G  R  G  Q  L  Q  *
                                                            M  K  T  A  S  K  F  S  V  M  F  F  M  L  L  A

 2701 CTCTGCGGCTGCTGGGATGTCAAAGATATCGAGCAACTATCCTTCGCCAGAGGGCTGGCGATCGATGAGACAAATGATCACCAGTACAAACTGACGTATC
        L  C  G  C  W  D  V  K  D  I  E  Q  L  S  F  A  R  G  L  A  I  D  E  T  N  D  H  Q  Y  K  L  T  Y  Q
```

```
2801  AAAATCTTCTTCCCCAAAGCGAAGACAGCCAAGCATCCGGAAAACCCGAATTTGTTAATGTAACGTCCCACGGGAAAACGATTCTGGAAGCAGTCAGTGA
        N  L  L  P  Q  S  E  D  S  Q  A  S  G  K  P  E  F  V  N  V  T  S  H  G  K  T  I  L  E  A  V  S  D

2901  TGTATCCATCAAGGACCCTCCTGTTTATAGCGATCATTTAAAAGTGATCCTTCTGGGAGAAAAACTGATGAGGAATCAAAATGTAGACCAAGTCCTCAAC
        V  S  I  K  D  P  P  V  Y  S  D  H  L  K  V  I  L  L  G  E  K  L  M  R  N  Q  N  V  D  Q  V  L  N

3001  CACTTTATCCGGGATGATGAGCTGCGGCGAAGCAGCTATTTGATGGCAGCCAGAGGGAATGCAGCTGATGTTTTCACAAAAGGAAACCCCAATCAGCAGC
        H  F  I  R  D  D  E  L  R  R  S  S  Y  L  M  A  A  R  G  N  A  A  D  V  F  T  K  G  N  P  N  Q  Q  Q

3101  AGCCGATGCCCTCCGAAAAACTGATTGATTTGACAACTCACAGCGGATATAACGGTAAGATTATGATACCGCTGCGCATCGGAAGAGCTTCTGTCTACTC
        P  M  P  S  E  K  L  I  D  L  T  T  H  S  G  Y  N  G  K  I  M  I  P  L  R  I  G  R  A  S  V  Y  S

3201  TCAGAACGGGTACAGTTATCTTATTCAAGCCGTGAAAAACGAAAAGGGAAAAGCCAAGTATGACGGAGCAGGCATTATCAAAAGGGGCAGCAATAAACTC
        Q  N  G  Y  S  Y  L  I  Q  A  V  K  N  E  K  G  K  A  K  Y  D  G  A  G  I  I  K  R  G  S  N  K  L

3301  GTTGGGTTTCTTTCAGCTGATGAAACCCAAACACTGTCATGGGTCATGGGGACGATCCAAGGCGGTGTCATGCCGACGACAGATAAAGGACATCCGATTA
        V  G  F  L  S  A  D  E  T  Q  T  L  S  W  V  M  G  T  I  Q  G  G  V  M  P  T  T  D  K  G  H  P  I  T

3401  CGTTTGAAATTAAAAAGTCGAAAACGAAAATTAAGCCCGTCATTGAAAACGGAAAGCCTGTCTTTCATATTTCTGTTAAAACGAAGGGCATCCTGACAGA
        F  E  I  K  K  S  K  T  K  I  K  P  V  I  E  N  G  K  P  V  F  H  I  S  V  K  T  K  G  I  L  T  E

                                                                                    HindIII
3501  AGACCAAAACCCGAATGAAAACTCCTTTAGCAAAAGTTATTTGCACAGGCTGGAAAACATTTTTGAAAAAAAGCTTGAACGAGATGTAAAGCAGGTGATG
        D  Q  N  P  N  E  N  S  F  S  K  S  Y  L  H  R  L  E  N  I  F  E  K  K  L  E  R  D  V  K  Q  V  M
           PstI
3601  GATAAACTGCAGCACGAGTATAAAACCGATCCGGTCTTTTTATCAGACCACATAAGGATTCAACACCCTGACTACTGGAATAAAGTAAAAGGGCATTGGG
        D  K  L  Q  H  E  Y  K  T  D  P  V  F  L  S  D  H  I  R  I  Q  H  P  D  Y  W  N  K  V  K  G  H  W  D

3701  ATGAAATATTTTCTGAGACTGATTTTAAGTACGATATTTCTTTTAAAATCATTAACTTTGGCACGGTGGGAAAGTAAGCAATCAAAAGGAGCGCGTGATC
        E  I  F  S  E  T  D  F  K  Y  D  I  S  F  K  I  I  N  F  G  T  V  G  K

3801  GCACCCTTTTTATGTTCCGACAGTCTGCT  3829
```

Fig. 2. Nucleotide sequence of the *gerB* region. The sequence of 3829 bp is derived from subclones shown in Fig. 1. There are three large ORFs which are shown below the sequence, each with a potential ribosome-binding site which is underlined. The operon also has a potential $E\sigma^G$-type promoter upstream of ORF 1, which is double-underlined. There is no obvious terminator after ORF 3.

of the ORF. The integration of subclone B into the wild-type gave a Ger$^+$ phenotype. This result may be misleading, as there is a possibility of readthrough from a plasmid-borne promoter with the vector, pMTL20EC, which may have yielded sufficient gene product to give the Ger$^+$ phenotype. This would not affect the result with C, as the ORF and plasmid are in the opposite relative orientation in this construct. A plasmid construct was made with DNA from subclone B cloned in the same vector in the opposite orientation. When integrated into strain 1604 this plasmid gave a Ger$^+$ phenotype, which is a further argument for operon structure. Whilst read-through from a plasmid promoter at sporulation may only give a low level of expression, this may be sufficient. The level of *gerA* expression is known to be low (Feavers *et al.*, 1990) and multiple promoterless copies of *gerA* ORFs are sufficient to complement *gerA* mutations (Zuberi *et al.*, 1985).

The pattern of results for plasmid integration into the mutant strains provides information as to the locations of the *gerB* alleles. The data show that the *gerB90*, *-92* and *-94* alleles all lie in *gerBA* as they are all corrected by subclone A, which only carries DNA from the *gerBA* region, but the alleles have distinct locations within the ORF as *gerB90* and *-94* are corrected by subclone B, which overlaps this region. As *gerB18* is not corrected by A but is corrected by B it can be deduced that the lesion lies in the last 50 codons of *gerBA* or in the first 296 codons of *gerBB*. Hence *gerBA* and *gerBC* have been shown to be required for AGFK germination, and it seems likely that *gerBB*, too, is necessary.

DISCUSSION

The sequence of the *gerB* spore germination operon has been elucidated and has revealed that *gerB* is a homologue of the *gerA* operon. In addition *spoVAF*, the sixth ORF in the *spoVA* operon (Fort & Errington, 1985), has a lower degree of homology with the *gerBA*/*gerAA* group. Furthermore *gerK*, mutations in which cause a defect in the AGFK response, has been shown to encode a further homologue of the GerAC/GerBC group (R. Irie, unpublished). GerK is more closely related to GerBC than to GerAC, but is not as close to either as they are to each other (B. Corfe, unpublished). Hence a family of proteins has now been identified, mostly involved in spore germination and produced in the forespore. Mutations affecting germination have been mapped in all three ORFs of the *gerA* operon and in at least two of the *gerB* operon (see Fig. 5), demonstrating the requirement of all these proteins for normal germination.

The highly conserved hydrophobic region in the GerAA/GerBA group probably indicates a region of functional importance. It is unlikely that this indicates a recent evolutionary divergence as the rest of the homology shows more divergence in both the hydrophobic and hydrophilic domains. Furthermore such a high level of identity between two hydrophobic domains that have not

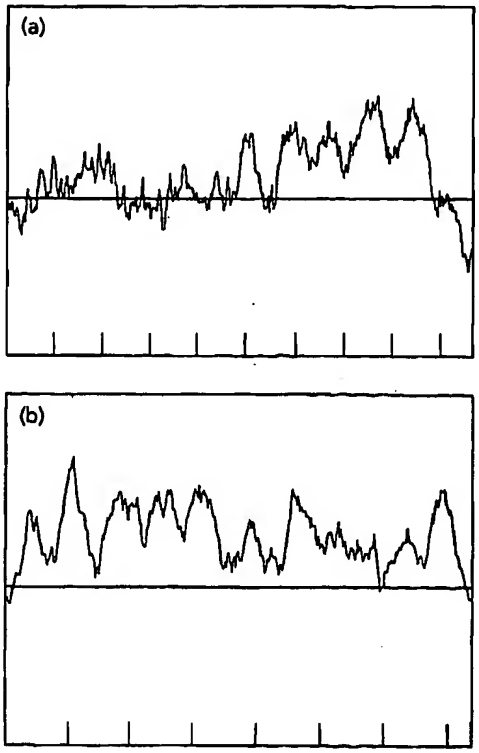

Fig. 4. Hydroplots of the GerBA and GerBB proteins. The *y*-axis shows increasing hydrophobicity, with the mean hydrophobicity for a large number of proteins represented by a horizontal line; the scale is from −4 to +4, according to the algorithm of Kyte & Doolittle (1982). Markers on the *x*-axes are at every 50 residues. The plots were calculated using a window size of 21. (a) The GerBA profile indicates an N-terminal hydrophilic domain, with possibly seven membrane-spanning hydrophobic segments and a smaller hydrophilic C-terminus. This pattern is similar to that found in GerAA. (b) The GerBB profile is highly hydrophobic, and is also very similar to its GerA counterpart. The pattern may suggest 10 or 11 transmembrane regions.

recently diverged is uncommon. This argues that this region of the protein must be particularly important in the function of the protein. As yet no possible functions have been individually assigned to any of the GerA/GerB proteins.

There are significant genetical and physiological differences between the ALA and AGFK germination responses. The germinant requirement is very different:

Fig. 3. Gap comparisons of the GerA and GerB proteins. The scores for percentage identity and conservative substitution for the pairs of proteins are as follows: 42/66 for GerAA vs GerBA (a); 31/60 for GerAB vs GerBB (b); 35/58 for GerAC vs GerBC (c). The most highly conserved region of the GerAA/GerBA pair is the hydrophobic domain between residues 200 and 400. This pattern of conservation is absent in GerAB and GerBB, which are predominantly hydrophobic proteins. The GerAC and GerBC proteins have homology to the prokaryotic lipoprotein attachment motif (underlined). Solid lines represent identical residues, double dots represent strongly conservative substitutions, and single dots represent weakly conservative substitutions.

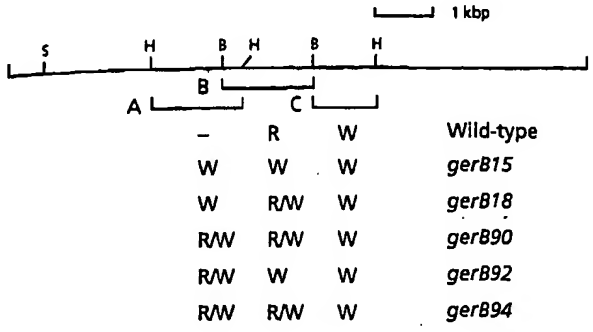

Fig. 5. Localization of the *gerB* alleles. The diagram shows the results of integration of three plasmid subclones into *gerB* mutant strains and the wild-type. Integration of subclones A or B into mutant strains may give rise to a mixture of Ger$^+$ and Ger$^-$ phenotypes if the subclone can correct the allele, depending upon whether recombination takes place to one side or the other of the lesion. The pattern indicates that *gerB90* and *gerB94* both occur in the region of overlap between A and B, *gerB92* occurs in the region of A not overlapped by B, and the converse for *gerB18*. Subclone A covers all of *gerBA* except for the last 50 codons. R, integrants all Ger$^+$; W, integrants all Ger$^-$; R/W, mixture of Ger$^+$ and Ger$^-$ phenotypes upon integration; B, *Bam*HI; H, *Hin*dIII; S, *Sph*I.

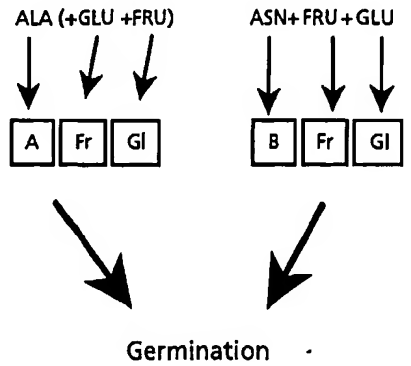

Fig. 6. Model for germinant receptors in the spore. The square boxes are *ger*-encoded proteins. The GerA-based receptor will respond to ALA alone, but the response will be improved by the presence of GLU and FRU stimulating the response via the appropriate gene products (which may be encoded by *gerD* and *gerK*). In the GerB-based receptor the additional gene products and germinants are obligatory for germination.

the ALA response depends upon a single amino acid as germinant (which is thought not to be metabolized, as non-metabolizable analogues of alanine will also trigger germination: Sammons *et al.*, 1981), and the response is not affected by metabolic inhibitors. Mutations in *gerA* are the only ones known to specifically block the ALA response without affecting the AGFK response. In contrast a complex set of germinants is required for the AGFK response, the response is inhibited by sodium azide (Venkatasubramanian & Johnstone, 1989) and mutants of *gerB* and *gerK* are specifically blocked in the response.

The *gerA* products appear to form a complete receptor/trigger unit in their own right, as the *gerA* operon is the only known operon in which mutation results in failure of spores to respond specifically to alanine. The *gerB* region encodes homologues of the complete ALA receptor/trigger mechanism, yet cannot trigger germination in the absence of the *gerD* and *gerK* gene products and further germinants. Spores of null mutants of *gerA* respond weakly to ALA with the GFK adjuncts, and this is dependent upon (at least) the *gerB* and *gerK* gene products (McCann, 1989). As GerK and GerD have been tentatively assigned the roles of glucose and fructose receptors respectively, it can be suggested that *gerB* encodes the ASN receptor which may also act as the secondary ALA receptor proposed by Sammons *et al.* (1981). The demonstration that *gerB* is a homologue of the probable principal ALA receptor/trigger reinforces this argument.

It is not obvious why the *gerB* products cannot stimulate germination in their own right. It now seems likely that ALA and AGFK germination occur by a more similar mechanism than could previously have been suggested on the basis of genetical and physiological evidence and it is therefore proposed that there are two similar types of germinant receptors in the spore. One may involve GLU and FRU receptors which are facultatively required to improve the response to ALA alone mediated by the *gerA* gene products. The other could have the GLU and FRU receptors working in obligate conjunction with an amino acid receptor, which is less fastidious than the ALA receptor and encoded by *gerB*. This model is summarized in Fig. 6. Whilst this model is novel in terms of the spatial arrangement and interactions between germination proteins that it implies, the mechanisms by which the germination apparatus function are still unknown and are the subject of our continuing enquiry.

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☐ **1: Cysteine Endopeptidases**

ENDOPEPTIDASES which have a cysteine involved in the catalytic process This group of enzymes is inactivated by sulfhydryl reagents. EC 3.4.22.
Year introduced: 2000(1988)

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- Cysteine Proteinase
- Cysteine Protease
- Acid Thiol Proteinase
- Neutral Thiol Proteinase
- SH Protease
- Sulfhydryl Proteinases
- Thiol Proteinase

- Thiol Proteinases
- Sulfhydryl Proteases
- Thiol Proteases

Previous Indexing:
- Peptide Peptidohydrolases (1966-1987)

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